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In vitro and in vivo Studies for Development of a Leishmaniasis Vaccine

ANNUAL REPORT

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The goal of this contract is to identify, characterize, and test antigens of Leishmania that could serve as candidate vaccines against cutaneous leishmaniasis. We successfully purified monoclonal antibodies from ascites by a protein G affinity chromatography method. These were employed to characterize antigens recognized by the monoclonal antibodies with Western blot methods. Attempts to assess whether the monoclonal antibodies were directed to carbohydrate moieties could not be undertaken since we found that antibodies non-specifically adsorbed metabolically-labeled antigens. Using a radioimmunoassay that we developed, we attempted to detect leishmanial antigens expressed on the surface of P388D1 cells infected with L. major.

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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SUMMARY

The purpose of the work supported by this contract is to identify antigens of Leishmania that could serve as candidates for an antileishmanial vaccine. During the contract period covered by this report (February 1,1988 - July 31, 1988) we succeeded in purifying antileishmanial monoclonal antibodies by protein G affinity chromatography and undertook studies to characterize antigens recognized by two purified monoclonal antibodies using Western blotting. The two monoclonal antibodies apparently recognized the same antigens that had Mr≅63 and 65kD. In contrast to our prior difficulties with non-specific binding of promastigote antigens to normal mouse IgG in immunoprecipitation assays, we did not experience this problem with Western blot techniques. In related studies, we attempted to label promastigotes with ^{14}C-glucose and ^{14}C-mannose in an effort to assess whether the epitopes recognized by the monoclonal antibodies were on glycoproteins or glycolipids. Finally, we used partially-purified monoclonal antibodies in an effort to detect leishmanial antigens expressed on the surface of infected P388D1 cells using a radioimmunoassay.

REPORT

The long-range goal of the research effort is development of a subunit vaccine for the prevention of cutaneous leishmaniasis. The strategy we are employing is to prepare monoclonal antibodies against Leishmania maior and utilize these in the immunoaffinity purification of parasite antigens. We are particularly interested in identifying those antigens that are expressed on the surface of infected macrophages, since we believe these to be the likely targets of lymphocytes that mediate defense in this disease.

The FIRST TECHNICAL OBJECTIVE for the period covered by this report (February 1, 1988-July 31, 1988) was to characterize the antigens recognized by the monoclonal antibodies of interest. To extend the initial efforts used in the prior budget period (February 1, 1987-January 31, 1988) which entailed immunoprecipitation of ^{35}S-methionine labelled promastigote lysates, we attempted to assess immunoprecipitation of material that labelled with carbohydrate substrates (glucosamine and mannose). The purpose of these efforts was to assess whether the monoclonal antibodies might recognize glycocongugates-- glycoproteins or glycolipids. The rationale for the studies was two-fold: 1) this approach might identify whether the proteins recognized by the antibodies were glycosylated; and, 2) this approach might detect glycolipids that would not be recognized by immunoprecipitation of ^{35}S-methionine labelled material.

The RESULTS of these experiments were discouraging. We labeled promastigotes with either ^{14}C-glucosamine or ^{14}C-mannose. We preadsobed labelled lysates with normal mouse Ig and reacted this with rabbit anti mouse Ig bound to protein A Sepharose to precipitate any non-specifically bound parasite material. As shown in Appendix 1, the autoradiograph of material that adsorbed to normal rabbit serum was qualitatively indistinguishable from that of material that bound to rabbit anti-promastigote polyclonal antibody. This problem was reiterated by the experience with normal mouse Ig; most of the labelled material (defined on the basis of counts per minute; cpm) was adsorbed when reacted with normal mouse Ig (Appendix 2). ^{14}C-mannose labelling resulted in

substantially greater incorporation into whole promastigotes, but the yield (cpm) of membrane lysates was too low to provide discernible bands on autoradiographs (Appendix 2).

The SECOND TECHNICAL GOAL was to purify monoclonal antibodies from ascites. In the prior budget period, we harvested several ascites samples and stored these in the presence of PMSF (a serine esterase inhibitor). We tried a number of purification methods during that period, but without success. The fact that all the monoclonal antibodies were IgG_3 isotype posed a special challenge, since this isotype of mice binds weakly to protein A, and because our ascites were heavily contaminated with transferrin and albumin (among other proteins) that were difficult to remove with ion exchange columns. We were particularly anxious to obtain purified antibody, in light of our experiences in working with ascites; proteins other than Ig bound to parasites and to parasite-infected macrophages.

Ascites harvests were generally stored individually, rather than pooled. We tested separate aliquots of ascites (stored at 4° C) for their reactivity to promastigotes in the ELISA assay. To our dismay, we found that ascites induced by the same hybridoma contained inconsistent titers, and that storage resulted in falling titer . The basis for the latter observation was ultimately identified. PMSF stock solutions were routinely diluted in isopropanol, and then subsequently diluted in buffer prior to adding to ascites. Inadvertently, one of the investigators added 10 fold more PMSF. We surmised that the loss of activity occurred as a result of alcohol precipitation of Ig. We therefore made several efforts to resolubilize the aggregates (for example, with 4M guanidine or glycine), but withou avail. We therefore were obliged to begin raising ascites once more. A few low volume ascites samples, not treated in this manner, were still available for use.

During a period of several months we attempted additional affinity purification schemes, including CM-Affigel Blue and anti transferrin in a two step purification scheme, but without satisfactory results. We were finally able to identify a method for purification of monoclonal antibody

from ascites using a streptococcal G protein affinity column. As shown in Appendix 3, elution of monoclonal antibody (IA-1 and ID-1) was achieved with 0.1M glycine. SDS-PAGE of the fall-through (non-bound) and eluted material confirmed that the eluate contained purified IgG (light and heavy chains) (Appendix 4). Thus, the tedious efforts to try to achieve antibody purification by conventional methods finally gave way to our application of a more recently developed method of affinity purification of antibody.

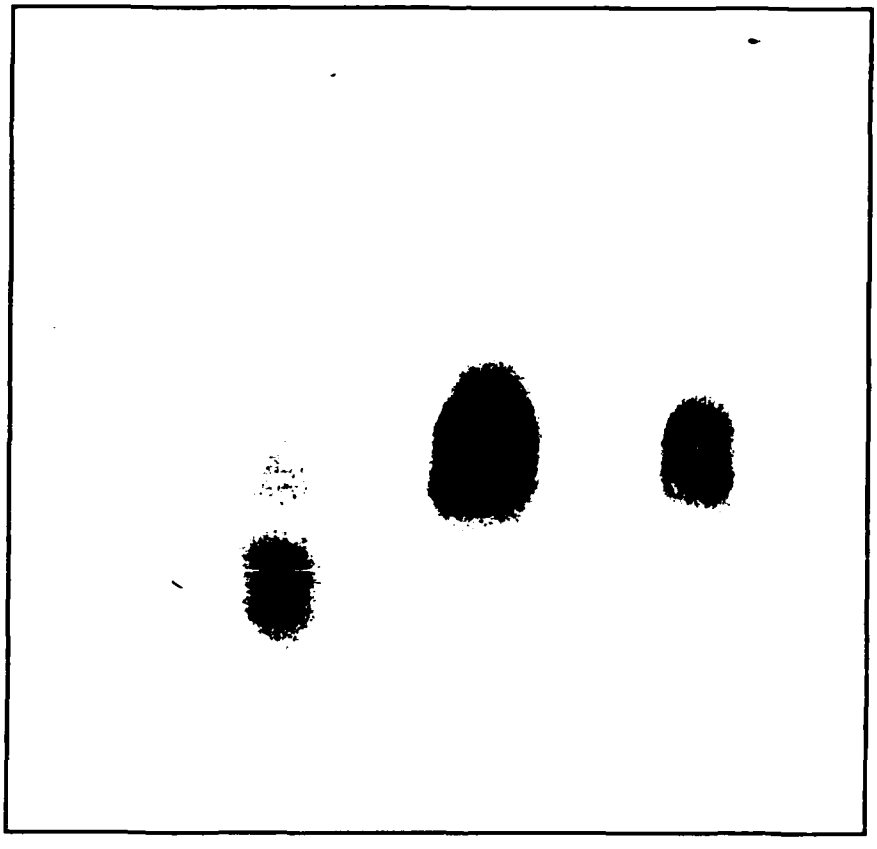
The THIRD TECHNICAL OBJECTIVE was to identify the antigens recognized by two purified monoclonal antibodies (IA-1 and ID-4), selected for their apparent superior ability to recognize parasite antigens expressed on the surface of infected macrophages (see Annual Report of first budget period).

The RESULTS are shown in Appendix 6 . Polyclonal rabbit and mouse antipromastigote antibody identified a variety of antigens when analysed by Western blot methods. Normal mouse Ig G identified none (contrast with immunoprecipitation results). IA-1 and ID-4 identified antigens of similar molecular weight. Since promastigotes also elaborate antigenic material into their culture medium, we performed Western blot analysis on these supernatants. As shown in Appendix 6, a greater variety of antigens were identified in the supernatants than in the membrane lysates. We could not convincingly distinguish the pattern of antigens identified by the two monoclonal antibodies, since the ID-4 antibody was less sensitive in revealing the bands than was IA-1, eventhough the two antibodies were tested at dilutions that gave comparable results in the antipromastigote ELISA. We interpret these findings to suggest that monoclonal IA-1 and perhaps also ID-4 identify a conserved epitope present in several of the antigens released by the promastigotes in culture. Since some of these antigens have higher Mr than antigens detected in the membrane lysates, we consider it unlikely that the multiplicity of antigens detected in the culture supernatants simply represents degredation of a few parent molecules.

The FOURTH TECHNICAL GOAL was to use monoclonal antibodies to identify antigens present on the the infected mecrophages. We used two-

step purified monoclonal antibody (ion exchange followed by antitransferrin antibody scrubber column) to assess binding to infected $P388D_1$ cells. As shown in Appendix 7, ascites containing monoclonal antibody ID-4 preferentially bound to infected rather than uninfected cells. Unfortunately, partial purification of antibody from ascites resulted in the loss of such preferential binding. This suggested that the second antibody used in this assay (rabbit antimouse Ig) probably also contained antibody to macrophage-binding constituents that were in ascites (such as transferrin). In any case, the observation pointed to the need for an improved purification scheme, which we identified (see above). Unfortunately, the contract period ended before we could test the streptococcal G protein-purified in the assay designed to assess antigens on the surface of infected cells.

Appendix 1: Immunoprecipitation of lysates of L.major promastigote that were metabolically labeled with 14 C-glucosamine.

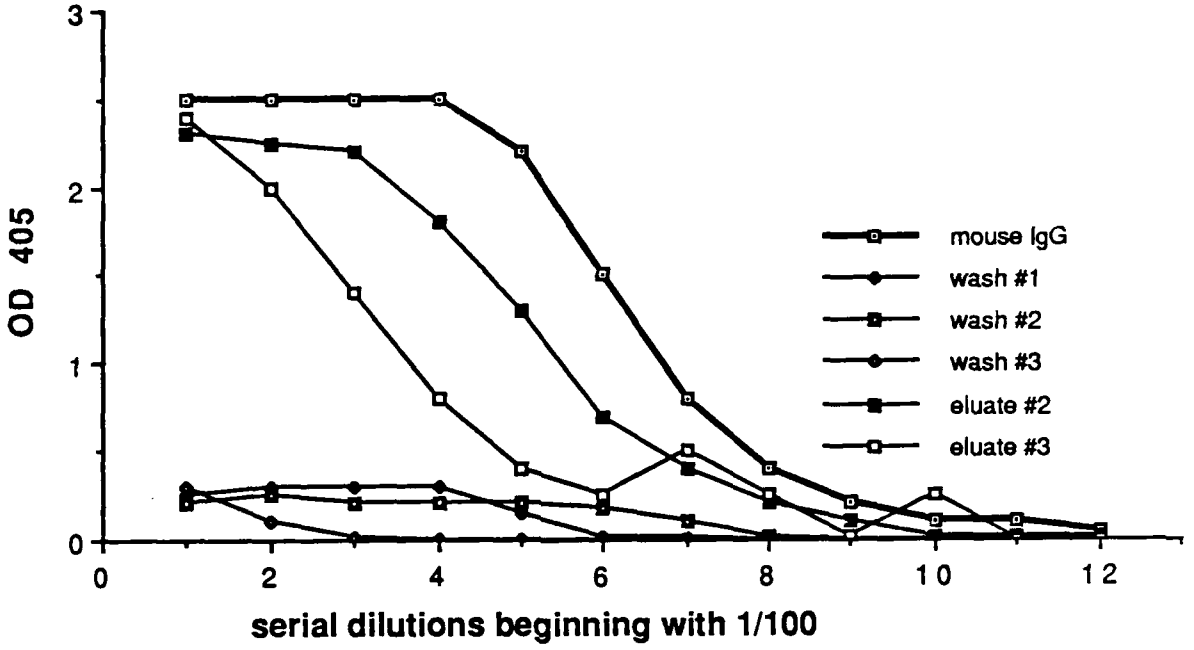


Lane 1, untreated lysate; Lane 2, lysate immunoprecipitated with normal rabbit Ig; Lane 3, immunoprecipitated with rabbit anti leismania polyclonal antibody.

Appendix 2: Immunoprecipitation of ^{14}C-glucosamine or ^{14}C-mannose labelled leishmania promastigote lysate by normal mouse IgG, rabbit polyclonal and mouse monoclonal antileishmanial antibody.

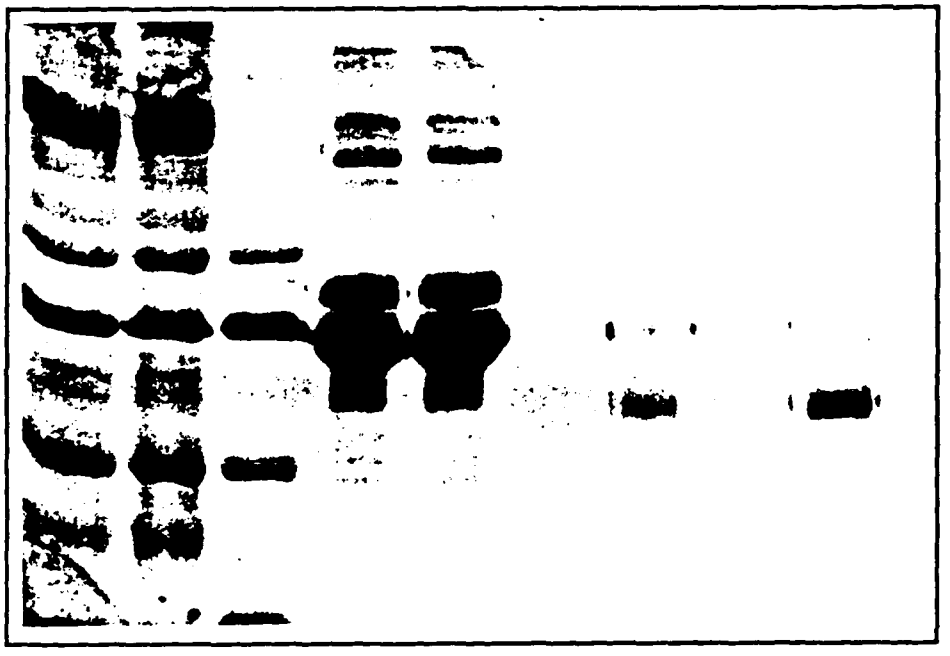
Treatment of sample with	CPM/ 25µL; substrate: glucosamine	mannose
normal mouse IgG		
sonicate	13,142	ND
lysate	2,971	85
rabbit polyclonal antibody		
sonicate	647	ND
lysate	522	350
monoclonal ID-4		
sonicate	375	ND
lysate	163	60
monoclonal IB-1		
sonicate	ND	ND
lysate	ND	150

Appendix 3: Purification of monoclonal antibody ID-1 by streptococcal protein G affinity chromatography.



Ascites was loaded onto a protein G-Sepharose Fast-Flow column that was equilabrated and washed with phospphate buffered (O.15M) saline. The bound material was then eluted with 0.1 M glycine-HCl, pH 3.0. Fractions were neutralised with Tris buffer and then dialysed extensively against PBS. These were tested for IgG content by ELISA, shown above. The IgG containing material was tested for reactivity to leishmanial antigens by Western blot (Appendix 4).

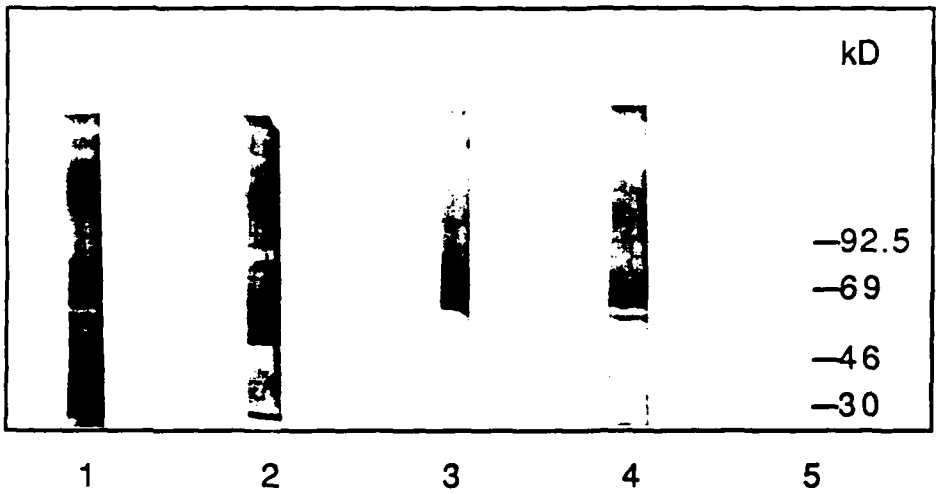
Appendix 4: SDS-PAGE of protein G-purified monoclonal antibodies ID-4 and IA-1.



1 2 3 4 5 6 7

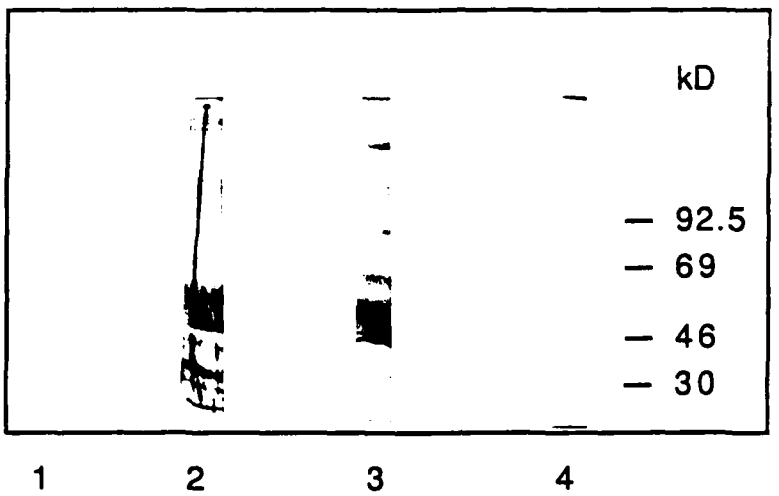
Ascites were treated by affinity chromatography on a column of Sepharose-conjugated streptococcal protein G fractions were subjected to SDS-PAGE (10%) under reducing conditions. Lane 1 and 2, high MW standards; lane 2, low MW standards; lane 3, fall-through of IA-1; lane 4, ID-4 fall-through; lane 5, glycine eluate of IA-1; lane 6, glycine eluate of ID-4. Heavy and light chains of IgG are discernible in lanes 6 and 7.

Appendix 5: Western blot analysis of L.major promastigote lysate.



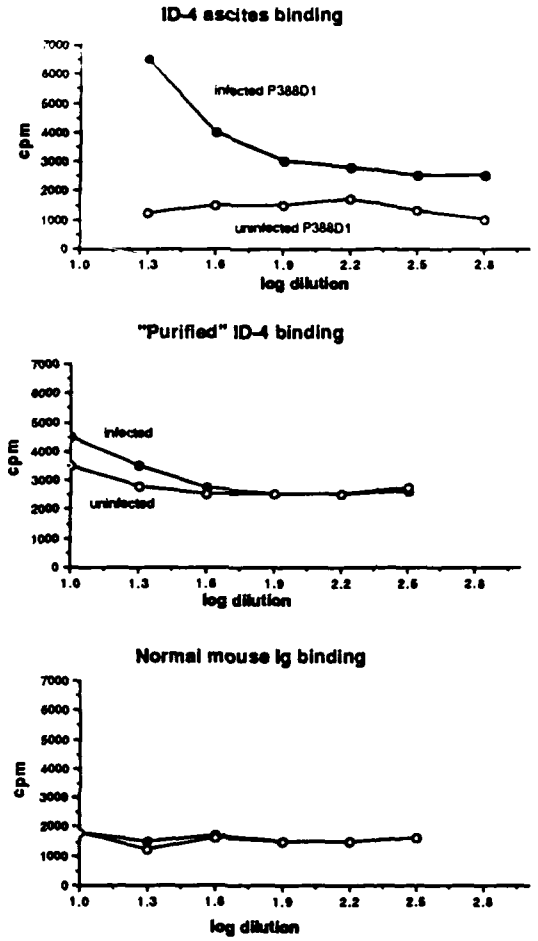
Polyclonal rabbit (lane 1), polyclonal mouse (lane 2), purified monoclonal antibody (IA-1 [lane 3]; ID-4 [lane 4]) and normal mouse IgG (lane 5) were used to probe electro-blots of promastigote lysate. Position of molecular weight markers is shown.

Appendix 6: Western blot analysis of L. major promastigote culture supernatant components separated by SDS-PAGE (reducing conditions; minigel).



Polyclonal mouse antileishmania antiserum (lane2), pre-immune mouse serum (lane 1), or purified monoclonal antibody IA-1 (lane 3) or ID-4 (lane 4) were used to probe electroblots of SDS-PAGE minigels of promastigote culture supernatants.

Appendix 7: Binding of monoclonal antibody ID-4 to L.major infected P388D1 cells.



P388D1 cells that were uninfected or infected 24h previously with L.major amastigotes were incubated with either 1)ascites containing antileishmanial monoclonal antibody (upper panel); 2) monoclonal antibody obtained by fractionating ascites on an ion exchange column followed by passage over an antitransferrin column (middle panel); or, 3) purified normal mouse IgG. Cells were then washed and treated with rabbit antimouse Ig, washed again, and treated with iodinated protein G. After the final washing, cells were placed in a gamma counter and cpm recorded. Mean of triplicate determinations are shown; SEM$\leq$10% mean in all cases.